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Research report

Atorvastatin attenuates the antinociceptive tolerance of morphine via nitric oxide dependent pathway in male mice

Mahsa Hassanipour[a,b,c,1], Hossein Amini-Khoei[a,d,1], Hamed Shafaroodi[e], Armin Shirzadian[a,b], Nastaran Rahimi[a,b], Muhammad Imran-Khan[a,b], Seyed-Mahdi Rezayat[b,e], Ahmadreza Dehpour[a,b,*]

[a] Experimental Medicine Research Center, Tehran University of Medical Sciences, Tehran, Iran
[b] Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[c] Department of Physiology and Pharmacology, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[d] Department of pharmacology, School of medicine, Kashan University of Medical Sciences, Kashan, Iran
[e] Department of Pharmacology and Toxicology, School of Pharmacy, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran, Iran

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ABSTRACT

The development of morphine-induced antinociceptive tolerance limits its therapeutic efficacy in pain management. Atorvastatin, or competitive inhibitor of 3-hydroxy-methyl-glutaryl coenzyme A (HMG-CoA) reductase, is mainstay agent in hypercholesterolemia treatment. Beyond the cholesterol-lowering activity, exploration of neuroprotective properties of this statin indicates its potential benefit in central nervous disorders. The aim of the present study was to assess the effects of atorvastatin in development and expression of morphine-induced analgesic tolerance in male mice and probable involvement of nitric oxide. Chronic and acute treatment with atorvastatin 10 and 20 mg/kg, respectively, could alleviate morphine tolerance in development and expression phases. Chronic co-administration of nitric oxide synthase (NOS) inhibitors including L-NAME (non selective NOS inhibitor; 2 mg/kg), aminoguanidine (selective inducible NOS inhibitor; 50 mg/kg) and 7-NI (selective neuronal NOS inhibitor; 15 mg/kg) with atorvastatin blocked the protective effect of atorvastatin in tolerance reversal. Moreover, reversing the atorvastatin effect was also observed in acute simultaneous treatment of L-NAME (5 mg/kg) and aminoguanidine (100 mg/kg) with atorvastatin. Co-treatment of guanylyl cyclase inhibitor, ODQ (chronic dose: 10 mg/kg and acute dose: 20 mg/kg) was associated with prevention of atorvastatin anti-tolerance properties. Our results revealed that the atorvastatin modulating role in morphine antinociceptive tolerance is mediated at least in part via nitric oxide in animal pain models of hot plate and tail flick.

1. Introduction

Morphine and related opioids, the most potent analgesic class, have been widely utilized for acute and chronic pain management. The clinical utility of opioids is a huge challenge for management of chronic pain such as cancer pain and neuropathic pain due to the rapid development of tolerance and hyperalgesia (Marek et al., 1991; Mao et al., 1995; Mayer et al., 1995; Trujillo and Akil, 1991). The loss of effectiveness after continued exposure or the requirement for dose increasing to maintain the same therapeutic effect is the definition of tolerance which occurs in two independent phases (induction and expression) (Bhargava, 1994; Johnson and Fleming, 1989; Raghavendra and Kulkarni, 1999).Therefore, restoring the efficacy of these major analgesics is of great importance in clinical setting.

Statins identified in 1976 by Endo and colleagues (Endo et al., 1976). These medications are competitive inhibitors of 3-hydroxy-methyl-glutaryl coenzyme A (HMG-CoA) reductase which catalyzes the conversion of HMG-CoA to mevalonate (an early, rate-limiting step in cholesterol biosynthesis) (Schachter, 2005). Statins are the best-tolerated and most effective agents for treating dyslipidemia (Zhou and Liao, 2009). Statins exert pleiotropic effects which could be protective in many conditions including neuro-

* Corresponding author at: Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, PO Box 13145–784, Tehran, Iran.
E-mail addresses: mhasanipoor@gmail.com (M. Hassanipour), aminikhoyi@gmail.com (H. Amini-Khoei), hshafaroodi@gmail.com (H. Shafaroodi), armin_shirzadian@yahoo.com (A. Shirzadian), nastaran.rahimee@gmail.com (N. Rahimi), muhammad_ik12@yahoo.com (M. Imran-Khan), rezayat1387@gmail.com (S.-M. Rezayat), Dehpour@sina.tums.ac.ir (A. Dehpour).
[1] The first two authors are considered as the first author.

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logical disorders, inflammation, pain and cardiovascular diseases (Bifulco et al., 2008; Ludman et al., 2009; Reiss and Wirkowski, 2009; Rosenson, 2001; Shi et al., 2011; Weitz-Schmidt, 2002; Zhou and Liao, 2010). Recently, several landmark experimental trials demonstrated the beneficial effects of statin therapy for morphine-induced analgesic tolerance and dependence. Mansouri et al. (2015) reported that simvastatin modulates morphine antinociceptive tolerance and physical dependence. There is evidence indicating that rosuvastatin attenuates the morphine analgesic tolerance (Li et al., 2015a,b).

Nitric oxide (NO), a potent guanylyl cyclase stimulator, is synthesized by nitric oxide synthase (NOS) from amino acid L-arginine via a NADPH-dependent pathway. The recognized isoforms of NOS are: inducible (iNOS), neuronal (nNOS) and endothelial (eNOS), the last two enzymes are constitutively expressed (Förstermann and Sessa, 2012; Shafaroodi et al., 2012, 2015). Based upon the previous reports NO and cGMP (*cyclic guanosine monophosphate*) systems mediate opioid antinociception and tolerace/dependence phenomena (Babey et al., 1994; Elliott et al., 1994; Vaupel et al., 1995).

Nitric oxide may play dual role in both phases of morphine tolerance. Studies suggest that NOS inhibition prevents naloxone-precipitated withdrawal signs and development and expression of analgesic tolerance while some reports indicate that inhibition of NOS accelerates the development of tolerance. (Dambisya and Lee, 1996; Kolesnikov et al., 1993). It is well-determined that blockade of NO overproduction through selective iNOS inhibitors attenuates the development of morphine tolerance and dependence and administration of NO donors leads to exacerbation of opioid withdrawal signs in dependent animals (Abdel-Zaher et al., 2006; Dambisya and Lee, 1996). Data about role of NO in tolerance and dependence phenomena are controversial.

It has been presumed that atorvastatin neuroprotection is not entirely due to cholesterol reduction but several mechanisms such as NO/cGMP pathway are responsible in part for the salutary effects. Statins rapidly enhance the NO bioavailability which augments cerebral perfusion, and up-regulates the eNOS expression via inhibition of isoprenylation of RhoA GTPase. Statins prolong the half-life of NOS, increase the expression of iNOS and nNOS, activate the eNOS via PI3K/protein kinase Akt pathway and lead to transcriptional alteration (Moezi et al., 2012; Stepień et al., 2005; van der Most et al., 2009; Wang et al., 2010).

The current investigation aimed to assess the role of atorvastatin in development and expression phases of morphine-induced analgesic tolerance by two pain models: hot plate and tail flick. We also examined the possible contribution of NO in atorvastatin effects on morphine analgesic threshold.

2. Materials and methods

2.1. Subjects

This study was performed on male NMRI (Naval Medical Research Institute) mice weighing 23–30 g (Tehran University of Medical Sciences, Iran). Animals were housed in the standard cages and maintained under controlled laboratory conditions (temperature: 24 ± 1 °C, humidity: 55 ± 10%, lighting: 12-h light/dark cycle) with free access to both standard laboratory pellet chow and tap water. All procedures were conducted in compliance with institutional Guideline for the Care and Use of Laboratory Animals with the approval of Tehran University Research and Medical Ethics Committees. All behavioral experiments carried out at the same time of the day. Each mouse was used only once in this study and each group consisted of 6-8 animals.

2.2. Chemicals

The following drugs were used throughout this study: atorvastatin, a HMG-CoA inhibitor (Sobhan, Iran); morphine, an opioid agonist (Temad, Iran); L-NAME [L-N^G-Nitro-L-arginine methyl ester hydrochloride], a non-specific inhibitor of NOS (Sigma, USA); aminoguanidine, a selective inhibitor of iNOS (Sigma, USA); 7-NI (7-nitro indazole), a selective inhibitor of nNOS (Sigma, USA); L-arginine, a NO donor (Sigma, USA) and ODQ [1H-[1,2,4] Oxadiazolo[4,3-a]quinoxalin-1-one], a selective and potent sGC (soluble guanylyl cyclase) inhibitor (Sigma, USA). Atorvastatin suspension was prepared in carboxymethyl cellulose (CMC, 0.5%) and was administered trice daily by oral gavage. Morphine, L-NAME, aminoguanidine, 7-NI and ODQ were given intraperitoneally (i.p.) in a volume of 10 ml/kg of the mice body weight based on two protocols which are defined in the treatment section. Morphine, L-NAME and aminoguanidine were dissolved in sterile isotonic saline solution. 7-NI and ODQ were prepared as fine suspensions in sterile isotonic saline using polysorbate 80 and DMSO 1% (w/v) as co-solvents.

2.3. Induction and assessment of morphine tolerance

Morphine analgesic tolerance was induced via repeated injection of morphine trice a day for 5 consecutive days: 50 mg/kg (8:00 a.m), 50 mg/kg (11:00 a.m) and 75 mg/kg (4:00 p.m, higher dose prevents the withdrawal signs over-night). On the 5th day animals received only a single dose of morphine 50 mg/kg (Dambisya et al., 1991; Homayoun et al., 2002; Javadi et al., 2013). Loss of antinociceptive property of morphine in hot plate and tail flick tests was used to assess the degree of tolerance. Hot plate test: animals were placed separately on an electrically-heated surface (55 ± 1 °C) (Tahghigh-Gostaran-Teb, Iran). To confine the mouse on the heated surface, an open plexiglas tube (18 cm high × 22 cm diameter) was used. The time interval (sec) between placement of animal and licking the hind paws or jumping with all four feet was recorded by a stopwatch as the end point. The increase in hot plate threshold was considered as a measure of analgesic activity. In order to avoid tissue damage animals were removed from the hot plate surface if they could not respond within 90 s. Animals showing a reaction time greater than 90 s were excluded from the subsequent experiment. In a trial, prior to drug administration animals were tested for 5 days in order to obtain a standard control reaction time level. The analgesic effect of morphine was determined 60 min after the first morphine injection on the first, third and fifth days. Tail flick assay: the apparatus (Ugo Basile, Italy) was used to measure response latencies. Animals were restrained with their tail positioned in tail flick apparatus. The light beam was focused on the dorsal surface of animal's tail. The time interval between initiation of radiant heat stimulation and abrupt removal of tail from this noxious stimulus was considered as tail flick threshold (cut-off time = 10 s) (D'amour and Smith, 1941). We applied the tail flick model 45 min after the first treatment on the first, third and fifth days. The two tests were performed on the same group of animals.

2.4. Treatments

To assess the role of atorvastatin in the induction and expression phases of morphine analgesic tolerance, we designed two protocols. In the protocol one: different doses of atorvastatin (0.01, 0.1, 5, 10, 20 mg/kg) were administered perorally (p.o) trice a day for 5 days 45 min prior to injection of every dose of morphine and hot plate/tail flick tests were performed on first, third and fifth days. In the second protocol: different doses of atorvastatin (0.01, 0.1,1, 5, 10, 20 mg/kg) were administered by gavage as single doses only on the 5th day 45 min before the last dose of morphine (50 mg/kg)

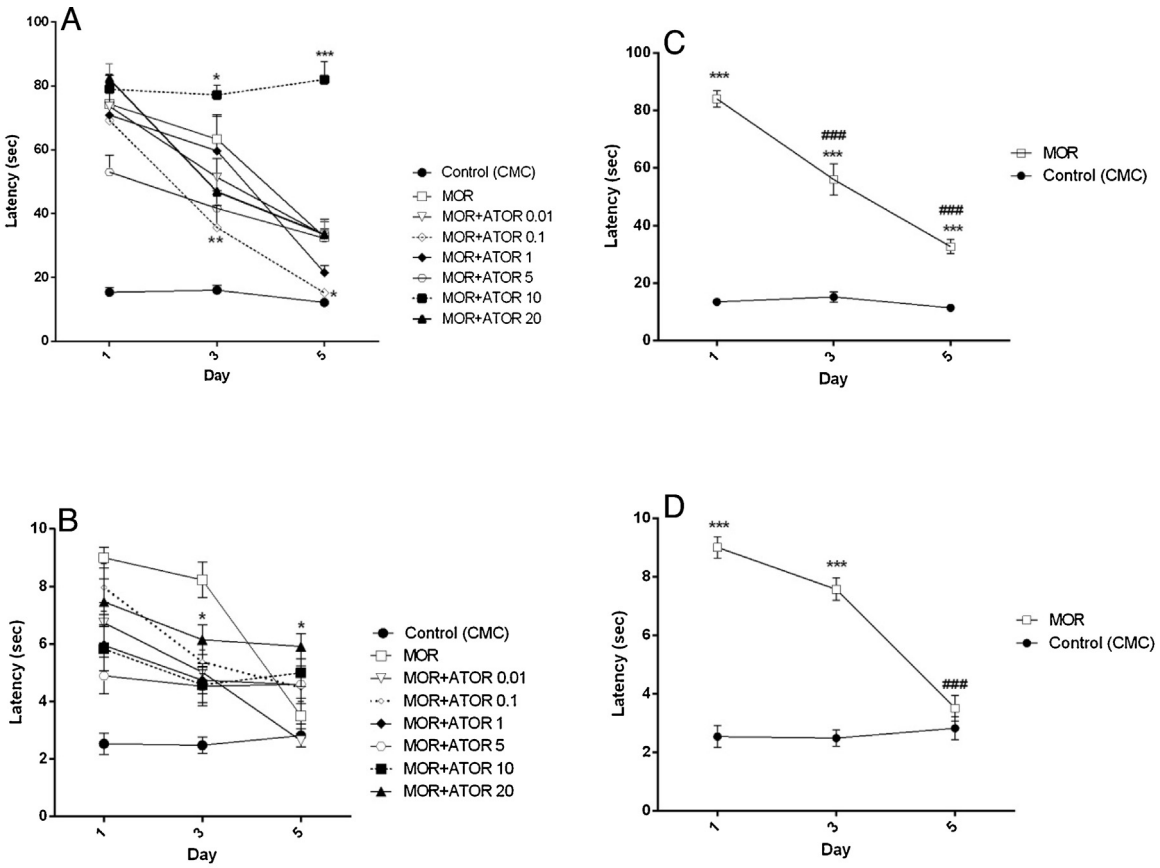

Fig. 1. The effect of chronic treatment with different doses of atorvastatin (ATOR) on morphine (MOR)-induced tolerance. ATOR was administered 5 days by gavage 45 min before MOR injection. Data are expressed as mean ± S.E.M of analgesic threshold of 6–8 mice. A: analgesic threshold assessment in hot plate test *P < 0.05, **P < 0.01 and ***P < 0.001 compared to morphine-treated group. B: analgesic threshold in tail flick test *P < 0.05 compared to MOR-treated group, according to one-way analysis of variance, followed by Tukey's *post hoc* test. C: Induction of morphine analgesic tolerance due to repeated injection of MOR during 5 days (hot plate). D: Induction of morphine analgesic tolerance due to repeated injection of MOR during 5 days (tail flick). ***P < 0.001 compared to control (CMC)-treated animals, ###P < 0.001 compared to MOR-treated animals on the day 1 according to two-way ANOVA repeated measure.

and analgesic tests were performed 1 h after morphine injection. In another experiment we administered these doses of atorvastatin (acute and chronic) alone 45 min before analgesic tests. To investigate the role of NO in the tolerance modulation by atorvastatin, NOS inhibitors and ODQ were administered to animals based on our two protocols. In protocol one: the NOS inhibitors including; L-NAME (2 mg/kg), aminoguanidine (50 mg/kg), 7-NI (15 mg/kg) and ODQ (10 mg/kg) were injected 30 min after each dose of morphine (75 min after atorvastatin) during the induction phase. Moreover; to determine the role of NO in the expression phase of tolerance L-NAME (5 mg/kg), aminoguanidine (100 mg/kg), 7-NI (40 mg/kg) and ODQ (20 mg/kg) and L-arginine 60 mg/kg (NO precursor) were administered only on 5th day 30 min after morphine injection (75 min after atorvastatin). Sub-effective doses of NOS inhibitors, ODQ and L-arginine were selected based on our previous studies (Hassanipour et al., 2016; Javadi et al., 2013).

2.5. Statistics

Data are presented as mean ± standard error of the mean (S.E.M). The one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons and two-way ANOVA repeated measure, followed by Bonferroni *post hoc* test were used to indicate the statistical significance of differences between the experimental means. P value < 0.05 was considered significant for all analyses. Graph-pad prism software version 6 was used to analyze the data of analgesic latency.

3. Results

3.1. Effect of chronic atorvastatin administration on the development of morphine analgesic tolerance

Fig. 1A illustrates the effects of chronic p.o administration of atorvastatin (0.01, 0.1, 1, 5, 10 and 20 mg/kg) on the analgesic threshold 45 min prior to morphine during 5 days. Atorvastatin (10 mg/kg) significantly (P < 0.05) increased the analgesic latency on day 3 with comparison to morphine-treated animals in day 3, ($F(2, 10) = 30.90$, $P < 0.001$), and also enhanced the threshold on day 5 compared to morphine analgesic effect on day 5, $P < 0.001$, ($F(7, 29) = 46.99$, $P < 0.001$). An atorvastatin dose of 0.1 mg/kg was associated with the analgesic threshold reduction compared to morphine analgesic latency and this effect was significant as a tolerance worsening effect, ($F(7, 28) = 16.55$, $P < 0.001$) for the third day and ($F(7, 29) = 46.99$, $P < 0.001$) for the fifth day. Fig. 1B illustrates that atorvastatin 20 mg/kg exerts a tolerance-reversal role ($P < 0.05$) in tail flick test, ($F(7, 40) = 11.40$, $P < 0.001$) and ($F(7, 41) = 5.267$, $P < 0.001$). In addition, other doses of atorvastatin exerted different patterns on the analgesic latencies in both models. The effective dose in hot plate and tail flick tests are different which reflects different neurologic mechanisms determined by each test. The tail flick test evaluates reflexive and spinally mediated response but

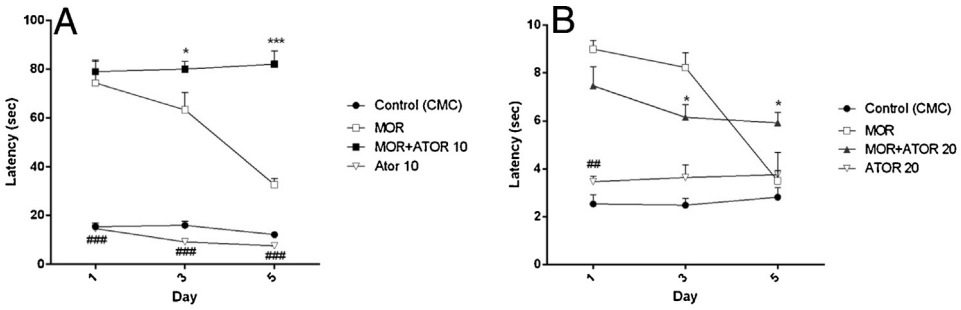

Fig. 2. The effects of different doses of chronic atorvastatin (ATOR) on pain latency following 5 days of atorvastatin administration. Various doses of ATOR were administered 90 min prior to analgesic tests, 2A for hot plate and 2 B for tail flick methods. Each point represents the mean ± S.E.M for 6–8 mice. *P < 0.05 and ***P < 0.001 to morphine (MOR)-treated animals. ##P < 0.01 and ###P < 0.001 compared to MOR + ATOR 20 and ATOR 10 + MOR-treated animals.

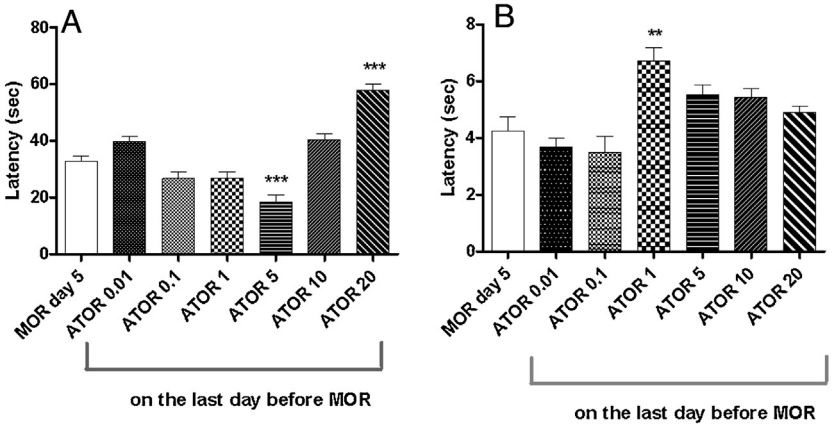

Fig. 3. The effect of acute treatment with different doses of atorvastatin (ATOR) on morphine (MOR)-induced tolerance. ATOR was administered 45 min before the last of MOR on day 5. Data are expressed as mean ± S.E.M of analgesic threshold of 6–8 mice. A: analgesic threshold assessment in hot plate test, ***P < 0.001 compared to morphine-treated group. B: analgesic threshold in tail flick test, **P < 0.01 compared to MOR-treated group, according to one-way analysis of variance, followed by Tukey's *post hoc* test.

hot plate method measures complex behaviors requiring neurological processing in the brain which leads to lifting/licking of a paw in response to the noxious stimulation (Mulder and Pritchett, 2004). The dose of 10 mg/kg was chosen for experiments allowing better detection of mechanisms of the observed effects. Fig. 1C and D show that during 5 days of morphine injection 3 times daily, tolerance is induced and the significant analgesic effect diminishes or disappears. Fig. C: in hot plate test ($F(5, 26) = 81.91$, $P < 0.001$) and Fig. D: in tail flick test ($F(5, 30) = 58.37$, $P < 0.001$).

3.2. Effect of different doses of chronic atorvastatin treatment on the analgesic threshold

We examined the effects of chronic atorvastatin treatment on analgesic threshold in order to exclude the effect of atorvastatin on analgesic latency. In several studies an analgesic effect for statins was reported (Garcia et al., 2011). So, we administered atorvastatin for 5 days 3 times daily to animals and then performed the hot plate and tail flick tests on the first, third and fifth days 45 min after atorvastatin treatment. Fig. 2A and B show that atorvastatin with the dose of 10 and 20 mg/kg could not induce an analgesic role per se in hot plate and tail flick respectively and the analgesic threshold is significantly lower than atorvastatin administration plus morphine on test days ($P < 0.001$). By these two experiments we can claim that morphine analgesic tolerance-reversal by atorvastatin is not related to analgesic effect of statin at the previously mentioned doses ($F(11, 57) = 54.04$, $P < 0.001$) and ($F(5, 27) = 7.301$, $P < 0.001$).

3.3. Effect of different doses of acute atorvastatin treatment on the expression of morphine analgesic tolerance

To clarify the role of acute atorvastatin in the expression of morphine tolerance, we administered different doses of this drug (0.01, 0.1, 1, 5, 10 and 20 mg/kg) only once 45 min before the dose of morphine on the last day. One-way ANOVA revealed a significant effect for atorvastatin ($F(6, 32) = 31.38$, $P < 0.001$) in hot plate test (Fig. 3A). Post-hoc analysis with Tukey's test revealed a significant tolerance blocking effect for acute atorvastatin at the dose of 20 mg/kg ($P < 0.001$) and a tolerance potentiating role for the dose of 5 mg/kg ($P < 0.001$). Fig. 3B shows that atorvastatin at the dose of 1 mg/kg could reverse the morphine induced tolerance ($P < 0.01$), ($F(6, 33) = 7.650$, $P < 0.001$) in tail flick method.

3.4. Acute atorvastatin treatment did not affect the analgesic threshold

The effect of acute atorvastatin administration on analgesic threshold is pictured in Fig. 4A/B. Atorvastatin at the dose of 1 and 20 mg/kg did not alter the analgesic threshold in comparison with control group. We administered these doses of drug 45 min before analgesic tests. These doses were selected based on the dose response which is clearly defined in Fig. 3. One-way ANOVA revealed a significant effect for morphine in elevating the threshold ($F(2, 17) = 161.2$, $P < 0.001$) (hot-plate, Fig. 4A) and ($F(2, 19) = 117.2$, $P < 0.001$) (tail-flick, Fig. 4B). *Post hoc* analysis with Tukey test showed that atorvastatin effect on the analgesic latency

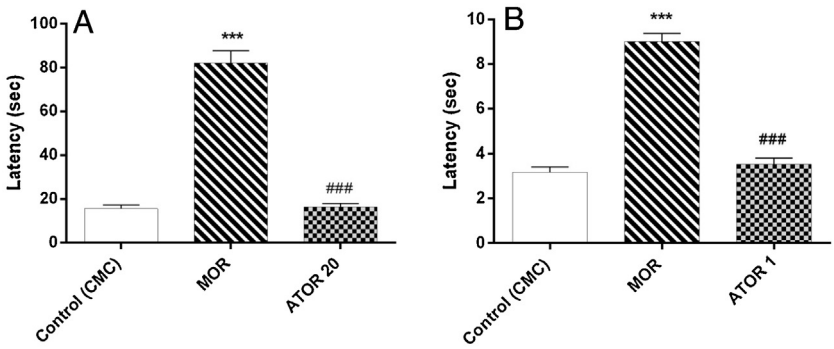

Fig. 4. The effect of different doses of acute atorvastatin (ATOR) administron on pain threshold. ATOR at the single doses of 20 and 1 mg/kg was administered 90 min prior to analgesic tests, 4A for hot plate and 4 B for tail flick method. Each point represents the mean ± S.E.M for 6–8 mice. ***P < 0.001 compared to vehicle (CMC)-treated animals, ###P < 0.001 compared to MOR-treated animals.

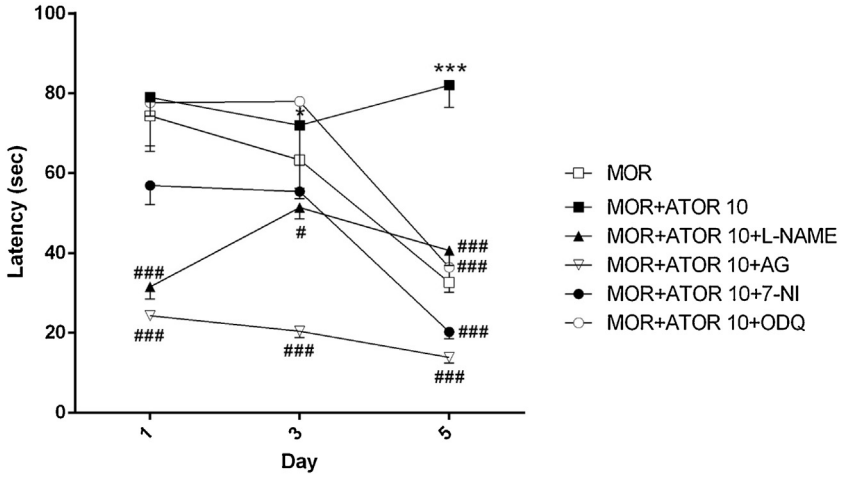

Fig. 5. The effects of chronic NOS inhibitors and ODQ administration on morphine (MOR)-tolerance reversal by atorvastatin (ATOR, 10 mg/kg) following 5 days of MOR tolerance induction. L-NAME 2 mg/kg, aminoguanidine (AG) 50 mg/kg, 7-NI 15 mg/kg and ODQ 10 mg/kg were injected 3 times daily 75 min after each dose of ATOR administration. Each point represents the mean ± SEM for 6–8 mice. * P < 0.05 and ***P < 0.001compared to MOR-treated animals, #P < 0.05 and ###P < 0.001compared to ATOR 10 mg/kg + MOR-treated animals, according to one-way analysis of variance, followed by Tukey's *post hoc* test.

at the above mentioned doses is significantly lower than morphine (P < 0.001) and the observed thresholds are in the range of control.

3.5. Attenuation of tolerance reversal with chronic atorvastatin administration by NOS inhibitors and ODQ in hot plate test

To investigate the role of NO in tolerance blocking effect of atorvastatin, L-NAME, a nonspecific NOS inhibitor, 7-NI, a specific nNOS inhibitor, ODQ, a specific sGC inhibitor and aminoguanidine, a specific iNOS inhibitor were injected 75 min after atorvastatin 10 mg/kg 3 times daily for a 5-day time span. As illustrated in Fig. 5, chronic administration of L-NAME 2 mg/kg and aminoguanidine 50 mg/kg completely reversed the tolerance blocking effect of atorvastatin in the third and fifth days. 7-NI 15 mg/kg and ODQ 10 mg/kg significantly blocked the protective property of atorvastatin only in the last day. Moreover, L-NAME 2 mg/kg, 7-NI 15 mg/kg, amioguanidine 50 mg/kg and ODQ 10 mg/kg per se did not alter the analgesic threshold (data not shown) and these doses are obtained based on our previous studies (Homayoun et al., 2002; Javadi et al., 2013).

3.6. NOS inhibitors and ODQ abrogated the tolerance blocking effect of acute atorvastatin at the dose of 20 mg/kg in hot plate test

Fig. 6 illustrates the effects of acute NOS inhibitors and ODQ co-administration with the protective dose of atorvastatin against

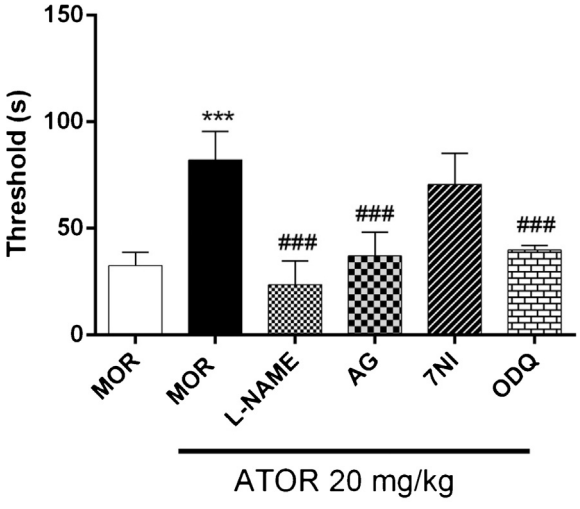

Fig. 6. The effects of acute NOS inhibitors and ODQ administration on morphine (MOR)-tolerane reversal by atorvastatin (ATOR, 20 mg/kg) following 5 days of MOR tolerance induction. L-NAME 5 mg/kg, aminoguanidine (AG) 100 mg/kg, 7-NI 40 mg/kg and ODQ 20 mg/kg were injected on the day 5, 30 min after the injection of the last dose of MOR. Each point represents the mean ± SEM for 6–8 mice. ***P < 0.001compared to MOR-treated animals, ###P < 0.001 compared to ATOR 20 mg/kg + MOR-treated animals, according to one-way analysis of variance, followed by Tukey's *post hoc* test.

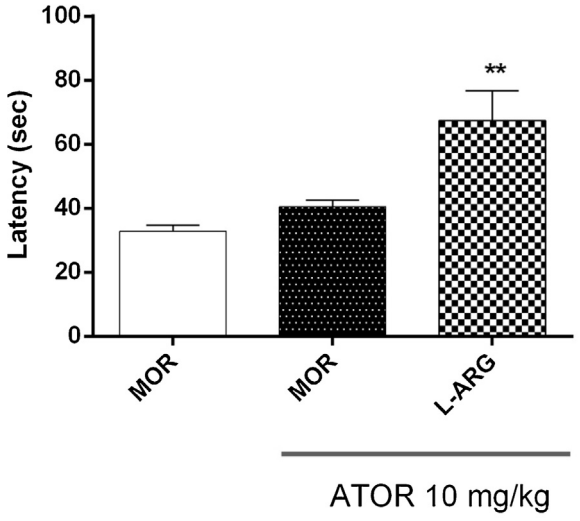

Fig. 7. The effect of L-arginine (L-ARG) on analgesic threshold elicited by hot plate test in animals treated with acute atorvastatin (ATOR) 10 mg/kg (single dose on day 5) and chronic morphine (MOR). L-ARG was injected 30 min after last dose of MOR. Data were expressed as mean ± S.E.M. N = 8; **P < 0.05 compared to MOR + ATOR 10-treated animals according to one-way ANOVA, followed by Tukey's *post hoc* test.

morphine-induced tolerance. Simultaneous administration of L-NAME 5 mg/kg, aminoguanidine 100 mg/kg and ODQ 20 mg/kg significantly ($P < 0.001$) blocks the tolerance reversing effect of atorvastatin 20 mg/kg based on One-way ANOVA ($F(5, 18) = 21.60$, $P < 0.001$). *Post hoc* analysis revealed that acute 7-NI administration did not inhibit the increased latency of analgesia induced by atorvastatin at dose of 20 mg/kg ($P > 0.05$).

3.7. Co-administration of acute L-arginine and atorvastatin 10 mg/kg enhanced the hot plate analgesic threshold in the expression phase

The effect of L-arginine, as a NO precursor, was investigated on the analgesic latency in the expression phase of morphine-induced tolerance. As shown in Fig. 7, simultaneous administration of L-arginine 60 mg/kg and subeffective dose of atorvastatin (10 mg/kg) led to significant ($F(2, 10) = 17.36$, $P < 0.001$) potentiation of the analgesic threshold ($P < 0.01$). L-arginine 60 mg/kg is considered as the subeffective dose based on our previous studies (Hassanipour et al., 2016).

4. Discussion

In the current study, we firstly observed that acute and chronic administration of atorvastatin by oral gavage totally blocked the existing morphine analgesic tolerance. Our further investigations proposed that NO system mediates the tolerance blocking effects of atorvastatin. Acute and chronic injection of L-NAME prevented the protective effect of atorvastatin in tolerance reversal. Besides that, acute and chronic aminoguanidine administration inhibited the analgesic threshold enhancement by atorvastatin. Moreover, in the development phase chronic 7-NI administration prevented the effects of atorvastatin, but its acute injection could not alter atorvastatin activity in the expression phase. Furthermore, acute and chronic administration of ODQ, a guanylyl cyclase inhibitor, prevented the protective effect of atorvastatin. In the animals received sub-effective dose of atorvastatin, acute treatment with L-arginine, a NO donor, augmented the morphine analgesic activity.

Opioids are still the last resort for moderate to severe pain but rapid development of analgesic tolerance (a profound loss of drug potency) remains as a frustrating problem (Ren et al., 2015). There are many hypotheses about how opioid tolerance develops, including, functional decoupling of opioid receptors from G-proteins which leads to receptor desensitization, phosphorylation of opioid receptors, internalization and/or down-regulation of μ-opioid receptor, upregulation of the cAMP (cyclic adenosine monophosphate) and PKC (protein kinase C) pathways, neuroimmune activation and neuroinflammation, glutamate homeostasis, nitric oxide production, modulatory role of ion channels or neurotrophic factors and activation of glial cells (astrocytes and microglia) at the level of the spinal cord (Ben-Eliyahu et al., 1992; Bian et al., 2012; Dang and Christie, 2010; DeLeo et al., 2004; Fukagawa et al., 2013; Koch and Höllt, 2008; Kolesnikov et al., 1993; Martini and Whistler, 2007; Ossipov et al., 2004; Vacca et al., 2013).

HMG-CoA reductase inhibitors (statins), known as common lipid-lowering drugs, have recently been shown to exert modulatory properties in pain management and morphine analgesic tolerance (Garcia et al., 2011; Li et al., 2015a,b). Li et al., 2015a,b showed that rosuvastatin delayed or partially reversed the morphine antinociceptive tolerance in rats. Considering the fact that chronic morphine treatment activates extracellular signal-regulated protein kinase (ERK) in both central and peripheral nervous systems leading to astrocyte activity as well as secretion of interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) and eventually central inflammation, they suggested that inhibition of ERK activation is one of the essential mechanisms.

Additionally, rosuvastatin has been found to restore analgesic effects of morphine after tolerance establishment during neuropathic pain treating in rats (Li et al., 2015a,b). Another studies indicated that simvastatin modulates analgesic tolerance and physical dependence in mice (Ghasemi et al., 2015; Mansouri et al., 2015). The data obtained in different studies demonstrates the beneficial effects of atorvastatin in decreasing the nociception and inflammation in animal pain models including writhing test, tail flick test and orofacial formalin test. Analgesic effects of atorvastatin have been mediated through several mechanisms including up-regulation the expression and activity of NOS, inhibition of cyclooxygenase-2 production, blocking the release of cytokine and prostaglandin, immunomodulatory role and possible direct analgesic action on nociceptors (Garcia et al., 2011; Kwak et al., 2000; Mosheimer et al., 2005; Santodomingo-Garzón et al., 2006).

In the present investigation, consistent with previous reports about the statin therapy benefits, atorvastatin administration (p.o, acute and chronic) could restore the antinociceptive effects of morphine following tolerance induction. The effect of atorvastatin on morphine analgesic tolerance is not dose dependent. Every dose has exerted a specific pattern and here is not a definite trend. Contrary to other reports, we did not observe any analgesic property for atorvastatin (without morphine) at the specific doses in both acute and chronic protocols in hot plate and tail flick tests. Since, the mechanisms underlying the protective role of statins on morphine tolerance inhibition have not been extensively clarified we studied the possible involvement of nitrergic system in this process.

There is considerable evidence that endogenous NO is involved in the modulation of morphine effects in various processes. It has been shown that L-arginine-NO pathway mediates pain and probably plays a modulating role in the antinociceptive responses to morphine (Brignola et al., 1994; Dambisya and Lee, 1995). Elevation in NO has been implicated in the induction of morphine analgesic tolerance and it was presumed that inhibition of NOS can attenuate the development of tolerance (Bhargava 1995; Heinzen and Pollack, 2004). Evidence suggests that 7-NI and aminoguanidine may impair the development of tolerance and also alters the downregulation of morphine responsivity (Abdel-Zaher et al., 2006; Heinzen and Pollack 2004).

Nitric oxide/cGMP pathway (especially at supraspinal site) plays an important role in modulation of opioids analgesia and

also morphine-sensitive nociceptive processes. Babey et al. (1994) showed that NG-nitro-L-arginine (NOArg) treatment prevents morphine tolerance and L-arginine accelerates it and when L-arginine was given alone decreased the potency of morphine. Thorat et al. (1993) suggested an important role for NO in development of κ-opiate tolerance. Przewłocki et al. (1993) reported that NO is involved in the spinal nociceptive events and elevated production of NO following the nociceptive input could diminish the efficiency of opioid antinociception in spinal cord. Dambisya and Lee (1996) demonstrated that NO has a role in both phases of morphine tolerance and dependence: increase in NO levels during the induction phase may delay the development of opioid tolerance/dependence while inhibition of NOS could accelerate the development of tolerance and inhibition of NO attenuates the expression of both tolerance and physical dependence. Xu et al. (1998) demonstrated that inhibition of supraspinal NO system prevents the development of acute morphine antinociceptive tolerance. However, enhanced production of NO did not affect the magnitude of morphine tolerance.

Based on the previous reports, a complex linkage between atorvastatin and NO production clearly exists (Amin-Hanjani et al., 2001; Laufs et al., 2000). Statin therapy modifies the NOS expression in pathological processes such as modulation of brain eNOS and inhibiting the cytokine-mediated upregulation of iNOS in astrocytes and macrophages during cerebral ischemia which lead to neuroprotection (Vaughan and Delanty, 1999). In case of *in vitro* studies, it has been demonstrated that statins prevent hypoxia mediated downregulation of eNOS which is regulated by Rho, the small GTPase protein, through the inhibition of geranylgeranylpyrophosphate synthesis or Rho kinase (the downstream target) (Endres and Laufs, 2004; Wang et al., 2010; Zhou and Liao, 2009). Studies indicated that atorvastatin treatment potently upregulates vascular nNOS mRNAs and protein via Akt/NF-κB signaling pathway, demonstrating a nNOS-mediated vascular effect for statins (Moezi et al., 2012; Nakata et al., 2007).

Our results showed that acute and chronic treatment of animals with L-NAME decreased the morphine analgesic efficacy which was elevated due to atorvastatin treatment in hot plate test. To further understand the effects of NOS isoforms, we examined the acute and chronic co-administration of iNOS and nNOS inhibitors with effective dose of atorvastatin. These results indicated that both iNOS and nNOS could reverse the enhanced analgesic threshold of morphine (arose from atorvastatin) in the development phase. However, in the expression phase we observed that only iNOS was responsible for atorvastatin potentiating effects in morphine analgesic latency. Acute co-administration of L-arginine with sub-effective dose of atorvastatin (acute) resulted in the enhancement of antinociceptive threshold of morphine.

The effects of NO may be both protective and harmful during morphine analgesic tolerance. NOS inhibitors themselves at effective doses could reverse the morphine tolerance, but in our study in order to investigate the possible mechanism for protective effect of atorvastatin in tolerance reversal, we selected the non-effective doses for NOS inhibitors and inducers based on our previous studies (Javadi et al., 2013; Homayoun et al., 2002). Our results showed that atorvastatin protective effects are possibly mediated by increasing the level of NO. This achievement is in line with Dambisya and Lee (1996) but in contrast with others.

An interesting finding in this study demonstrated that the potent and selective inhibitor of NO-sensitive guanylyl cyclase (ODQ) could completely inhibit the protective effects of atorvastatin therapy in both development and expression phases of morphine tolerance. Nitric oxide pathway exerts several of its physiological actions with soluble guanylyl cyclase stimulation (Moro et al., 1996). Guanylyl cyclase is an intracellular receptor for gaseous ligand (NO) which induces the generation of cGMP, protein kinase G (PKG) phosphorylation and changes in the activity of effector proteins such as phosphodiesterases, ion pumps and ion channels (Dupont et al., 2014).

In summary, our data reported that atorvastatin could be used along with morphine to restore morphine analgesic properties during development and expression phases of tolerance, suggesting a novel approach in the treatment of morphine analgesic tolerance which is clinically important. Moreover, we also pointed out for the first time that NO/sGC/cGMP signaling pathway contributes to alleviating effects of atorvastatin in morphine analgesic tolerance.

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